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Bioactivity of pyocyanin of *Pseudomonas aeruginosa* clinical isolates against a variety of human pathogenic bacteria and fungi species

Zahraa Jaafar Jameel[1], Anaam Fuad Hussain[2], Muthana Abdulkhader Al-Mahdawi[1], Nuha F. Abed Alkerim[3], Eman Sabah abd Alrahman[1]

1 Biology Department. College of Science. Diyala University, Iraq.
2 Biotechnology Department. College of Science. Diyala University, Iraq.
3 Department of chemistry, College of Science,Diyala University, Iraq.

Contact information:

Dr. Zahraa Jaafar Jameel.

dr.zahraa@sciences.uodiyala.edu.iq

Abstract

Objectives: Pyocyanin is blue pigment redox active, secondary metabolites produced by *P. aeruginosa.* The present study investigated the bioactivity of pyocyanin against certain types of bacteria and fungi causing human infections

Methods: A total of 23 clinical isolates of *P. aeruginosa* were collected from patients admitted to the General Baqubah Hospital during the period from November 2016 through May 2017. All isolates were cultured on Pseudomonas agar and confirmed by biochemical tests as *P. aeruginosa*. Pyocyanin extraction was done by chloroform method and concentration was determined by multiplying the optical density at 520 nm by 17.072 expressed as µg/ml. Biological activity of pyocyanin was determined by well diffusion procedure.

Results: According to the source of *P. aeruginosa*, the most tested isolates were from ear infection (30%) followed by wounds (22%), burns (17%), urine (13%) and both stool and diabetic leg ulcer (9%). Antimicrobial resistant of *P. aeruginosa* isolates were the following: 19 (82.6 %) to piperacillin, followed by 10 (43.5%) to aztreonem, 8 (34.8%) to meropenem, 6 (26.1%) to amikacin, 5 (21.7%) to ciprofloxacin, 2 (8.7%) to cefotaxime.The urine isolates produced the largest amount of pyocyanin (15.894 µg/ml). Pyaocyanin have shown antimicrobial activity against the following bacteria: *Shigella spp., Staphylococcus aureus* and *Staphylococcus epidermedis*. Also, pyaocyanin can inhibit the following fungi and yeast: *Aspergillus niger, Penicillium*

spp., Rhizopus spp, Trichophyton mentagrophyte, Rhodotorula spp., Alternaria alternate, Trichophyton rubrum and *Candida spp.*

Conclusions: Antimicrobial activity of pyocyanin was more observed against Gram-positive than Gram-negative bacteria, but mostly similar against all fungi (molds and yeast). Cefotaxime was the most active antimicrobial drug against all *P. aeruginosa* isolates

Keywords
Pseudomonas Aeruginosa, Pyocyanin, Antimicrobial Activity, Iraq.

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Introduction

Pseudomonas aeruginosa is an opportunistic pathogen of human and can often lead to skin infections, otitis externa, septicemia, necrotizing pneumonia, burns and surgical wounds infections [1, 2].

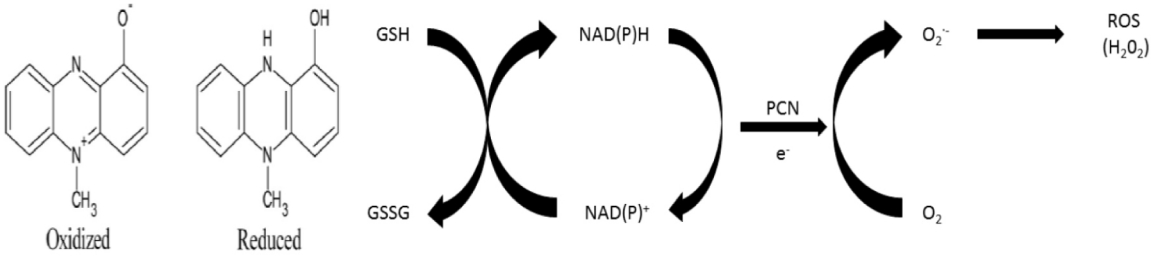
P. aeruginosa is an opportunistic pathogen and it is a frequent cause of nosocomial infections, many of which are responsible for high mortality among hospitalized patients in intensive care units (ICU) and immunocompromised patients even when treated with appropriate antibiotics [1-2].

Pseudomonas aeruginosa produces a variety of cell-associated factors and toxin, some of which are responsible for its invasion and the severity of diseases. These include lipopolysaccharide (LPS), phospholipase, proteases, flagella, pili, exotoxins and pyocyanin [3-4].

Pyocyanin is a blue redox active, secondary metabolite,phenazine derivative that it exhibits a paradoxical pro-oxidant property [4]. A zwitterions that canenterbiological membranes, pyocyanin can directly accept electrons from reducing agents such asNADPH and reduced glutathione **(Figure 1)** and endure redox cycling using oxygen as an electron acceptor resulting in the generation of the reactive oxygen species (ROS), particularly hydrogen peroxide, with resulting induction of oxidative stress and disturbance of intracellular redox homeostasis [5]. Therefore, the important mechanisms of pyocyanin to prokaryotic and eukaryotic cells are related to inhibition cellular respiration [6]

Pseudomonas spp. are also capable of producing organic volatiles, whose in vitro antifungal nature has been demonstrated against *Phytophthora-*

Figure 1: Chemical structure of reduced and oxidized forms of pyocyanin and mechanizm [5].



vignae in cowpea [7]. These compounds inhibited sclerotic and ascosporic germination, and mycelial growth of *Sclerotinia sclerotiorum*, in vitro and in soil tests [8].

This study investigated the bioactivity of pyocyanin produced by fresh *P. aeruginosa* clinical isolates from patients against a variety of bacteria and fungi causing human infections.

Material and Methods

Bacterial strain isolation and identification

This study was performed from November,2016 until May, 2017. A total of 23 *P. aeruginosa* isolates were collected from urine, stool, diabetes leg, ear, burns and wounds infections of patients admitted to General Baqubah Hospital. The isolates were recovered from cultures on Pseudomonas agar, MaCconkey agar and blood agar. All isolates were identified as *P. aeruginosa* using gram-stain, blue-green pigmentation and biochemical characterization [9].

Antibiotic susceptibility testing

The antimicrobial susceptibility assay was performed on Mueller-Hinton agar using disc-diffusion method, and selection of antibiotics and growth inhibition zones were interpreted according to the Clinical Laboratory Standards Institute [11]. The antimicrobial disks: piperacillin (100µg), cefotaxime (30µg), meropenem (10µg), aztreonem (30µg), amikacin (30µg) and ciprofloxacin (5µg) were obtained from (Mast Group, UK).

Pyocyanin Production

Nutrient broth was used for the extraction of pigment from each *P. aeruginosa* isolate after incubation for 2-3 days at 37°C. The change in color of the pigment to bluish green indicated pigment production [9].

Pyocyanin extraction

After the color media became bluish, 5 ml of each sample was centrifuged (3000rpm/10min) and 3ml of chloroform added to the supernatant and mixed until blue color is recovered. It was further confirmed by adding 0.2 N HCl to the blue color solution which turned to red– pink [12].

Pyocyanin quantitative assay

The absorbance of each recovered solution was measured at 520 nm. Pyocyanin concentrations were expressed as micrograms of pigment per milliliterof culture and were determined by multiplying theoptical density at 520 nm by 17.072, according to the absorbance of pyocyanin at 520 nm in acid solution. [13].

Pyocyanin purification

0.4 M borate-NaOH buffer (pH= 10) was added to the pink solution until the color turned to blue and again extracted by chloroform. This step was repeated 2 to 3 times resulting pyocyanin in chloroform in a clear blue solution. Finally pyocyanin powder was collected by evaporating the chloroform and weighted then rehydration by 1ml of sterilized distilled water [14].

Biological activity of recovered pyocyanin

The assay of antibacterial and antifungal of pyocyanin was carried out in well diffusion technique [15,16]. Wells with diameter size 6mm were made using Mueller-Hinton agar petri dish. Each one isolate of the following pathogenic bacteria were tested: *Escherichia coli, Klebsiella pnemoniae, Shigella, Proteus spp., Acinetobacter spp., Staphyllococcus aureus* and *Staphyllococcus epidermedis*. pathogenic fungi and yeast namely *Aspergillus niger, Penicillium spp., Rhizopus spp, Trichophyton mentagrophyte, Rhodotorula spp.*, *Alternaria alternate*, *Trichophyton rubrum and Candida spp*. The growth of each isolate was swabbed on the surface of nutrient agar and 100µl of pyocyanin solution (167mg/ml) were

added in to the well. Petri dishes were incubated at 37°C for 24-48 hours for bacteria and yeast, while kept for other fungi for up to one month. The inhibition zone in each well was measured by a ruler in mm unit to determine the biological activity of pyocyanin.

Results

A total of 23 *P. aeruginosa* isolates were investigated for production of pyocyanin. Distribution of *P. aeruginosa* isolates according to clinical source are shown in **Table 1**. The majority of isolates were collected from era infections (30%), followed by wounds (22%), burns (17%), urine (13%) and each leg diabetes and stools (9%).

Suscptibility to antibiotics

The results of antimicrobial susceptibility test of 23 *P. aeruginosa* isolates have shown high resistance rate (82.6 %) to piperacillin, followed by (43.5%) to aztreonem (34.8%), to meropenem (26.1%) to amikacin, (21.7%), to ciprofloxacin and (8.7%) to cefotaxime, respectively **(Table 2)**.

Table 1. Distribution of *P. aeruginosa* isolates according to clinical source

Source	*P. aeruginosa* isolates No (%)	%
Ear	7	30
Wounds	5	22
Burns	4	17
Urine	3	13
Leg Diabetes	2	9
Stool	2	9
Total	23	100

Table 2. Antimicrobial resistance profile of 23 *P. aeruginosa* isolates

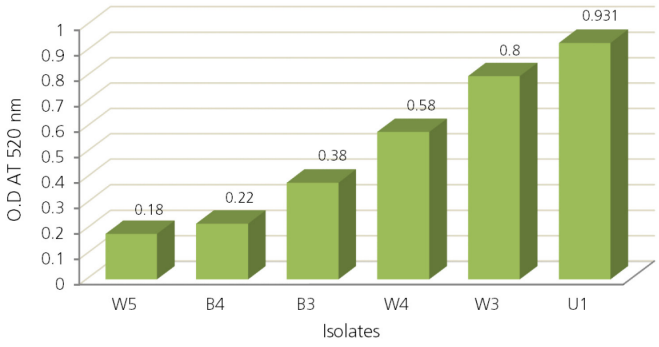
Antibiotic	Resistant %
Piperacillin	82.6
Aztreonem	43.5
Meropenem	34.8
Amikacin	26.1
Ciprofloxacin	21.7
Cefotaxime	8.7

Pyocyanin extraction (quantitative assay)

A total of 6 *clinical P. aeruginosa* isolates were selected to extraction of pyocyanin according to intensity of blue pigmentation on tested agar.

Figure 2, shows the amount of pyocyanin obtained from each *P. aeruginosa* isolate according to their clinical source. The largest amount of pyocyanin (15.894 µg/ml) was recovered from one urine isolate.

Figure 2: Pyocyanin quantity (concentration (µg/ml)=O.D. 520 nm ×17.072).



Wound isolate: W5, W4, W3; Burns isolates: B3, B4; Urine isolate: U1.

Biological activity of pyocyanin

The antimicrobial activity of purified pyocyanin (Needle-like crystal) at the concentration (167 mg/ml) was monitored against different microorganisms as shown in **Table 3**.

Gram-positive such as *Staphylococcus epidermidis, Staphylococcus aureus*; Gram-negative such as *Escherichia coli,proteus spp., Klebsiella spp.* and *Acinetobacter spp.*; molds such as and Yeast as *Candid aalbicans*. Results shown in table (3) indicate that pyocyanin shows high biological activity against all mentioned organisms, except against *Klebsiella pneumonia*, *Acinetobacter spp.*, *Proteus* and *Escherichia coli*.

Table 3. Diameter of antimicrobial activity of pyocyanin to various tested microorganisms.

Microorganism	Properties	Inhibition zone (mm)
Shigella spp.	Gram negative	25
Staphylococcus epidermedis	Gram positive	23
Staphylococcus aureus	Gram positive	23
Klebsiella pneumonia	Gram negative	None
Acinetobacter spp.	Gram negative	None
Proteus spp.	Gram negative	None
Escherichia coli	Gram negative	None
Penicillium spp.	Mold	25 (inhibition of spore formation)*
Aspergillusniger	Mold	25
Aspergillus spp.	Mold	23
Trichophytonmentagrophyte	Mold	23
Trichophytonrubrum	Mold	23
Rhizopus spp.1	Mold	23
Alternaria alternate	Mold	23
Rhodotorula spp.	Mold	23
Rhizopus spp.2	Mold	10
Candida spp.1	Yeast	20
Candida spp.2	Yeast	17
		*: Apply for all tested fungi.

Discussion

Pyocyanin is a blue pigment redox active produced by *P. aeruginosa*, The present study investigated production, purification, characterization and bioactivity of pyocyanin pigments produced by local clinical *P. aeruginosa* isolates. The results have shown that *P. aeruginosa* isolates were most commonly associated with ear infection (30%) followed by other body sites in our region of Iraq. Many studies carried out in Iraq and other countries demonstrated wide spread of *P. aeruginosa* isolates in clinical specimens. A study of Abbas in Baqubah/Iraq, has found that *P. aeruginosa* first commonly isolated from burns cases (18.18%), followed by ear (11.6%), urine (10.8%)and wounds (6.25%) [17]. While the study of Al-Imari in Baghdad/Iraq, has reported the incidence *P. aeruginosa* isolates from certain clinical specimens as follow: urine (22.4%), ear (18.4%), sputum (16.3%), burns (14.3%) and wounds (12.3%) [18]. El fouly and coworkers in Cairo, Egypt found that *P. aeruginosa* was the most common cause of urinary tract infection (12.5%), followed by burns (10%) and sputum (9%) [19]. The reasons of variations in the occurrence rates of *P. aeruginosa* infections may be due to underlining diseases of investigated patients, virulence factors and the local antimicrobial resistance of the organism, and other health conditions [2]. In addition, *P. aeruginosa* is able to act as opportunistic pathogens in human and patients with weakened immune systems, and especially during hospitalization in intensive care units [1-2].

The present study indicated that *P. aeruginosa* isolates have the highst resistant rate to piperacillin (82.6%) and less to other antimicrobial agents as shown in **Table 2**. The study of Al- Alkhozai and Alkabei in Diwanyia, Iraq, reported similar resistance rate in *P.aeruginosa* isolates to piperacillin (94%) and high resistance rate to aztreonem (88.5%) [20]. This study found a resistance rate of 43.5% to aztreonem, whereas the study in Baqubah/Iraq reported very low resistance rate of 5% to aztreonem among *P. aeruginosa* isolates [17]. The study of Ameen and coworkers in Pakistan, have found that *P. aeruginosa* resistant to aztreonem was (86.1%) [21]

It is well established that the development of resistance of *P. aeruginosa* to antibiotics is increasing locally and globally due to the overuse of antibiotics [1]. There are various multiple mechanisms contribute to developing antimicrobial resistance in *P. aeruginosa*, including active efflux, acquisition of various beta-lactamases,decrease outer membrane permeability and target modification [22-23]. Therefore, distinguishing the trends in resistance of *P. aeruginosa* becomes important for choosing the right antibiotic [24].

The production of pyocyanin is exclusive to *P. aeruginosa*, and its production can be improved by

culture strains on King's A medium which has potassium and magnesium salts in appropriate concentration to defeat fluorescein production (pyoverdine) [2]. Pyocyanin is blue color in alkaline and neutral pH while red color in acid pH. Any change in the pH may affect the color of pyocyanin [25]. In addition, the variation in pyocyanin production among different strains could be due to various factors such as lighting conditions. It has been reported that the concentration of pyocyanin was reduced under certain lighting conditions. This decrease was dependent on both the light intensity and wavelength and occurred with light in the ultraviolet and violet region of the spectrum [26-27].

The present study has demonstrated that pyocyanin production by all investigated clinical isolates had antimicrobial activity against Gram-positive *Staphyloccocus spp*. more than gram negative bacteria and to some extent against yeast such *Candida spp.* and filamentous fungi. This result is in agreement with those results of Makarand et al. (2007) which have reported that phenazine antibiotic (chemical substance similar to pyocyanin) have antimicrobial activity against strains of *Bacillus subtilis* and *Candida albicans* [28].

The study of El fouly in Egypt, found that *P. aeruginosa* isolated from human urine sample produced more pyocyanin concentration than isolates from rice cultivated soil (production titer was 6.3 and 5.9 mg/ml, respectively) [19], While Ra'oof and Latif, in Iraq, has detected that the *P. aeruginosa* isolate from sputum produced the largest amount of pyocyanin (12.069 µg/ml) [29]. The study of Charyulu *et al.* (2009), has shown that the secondary metabolite from Pseudomonas which played a significant role in lyses the pathogenic bacterial including MRSA strains [30]. Also, the Sudhakar *et al.* (2013) showed different antimicrobial activity against *E. coli, S. aureus, Proteus sp.,* and *Klebsiella spp.* [31], while the study Karpagam et al., (2013) found the pigment produced by *P. aeruginosa* has antifungal activity against *Candida* sp. and *Cryptococcus neoformans* [32].

In conclusion, pyocyanin produced by clinical *P. aeruginosa* isolates has various spectrum of antibacterial and antifungal activity against certain Gram-positive and Gram-negative bacteria, *Candida spp.* and filamentous *in vitro*.

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